Extended Experimental Procedures

Cells, viruses and reagents

Human colon cancer cell line HT-29, Vero, 293T, and 3T3-SA were maintained at 37º C in 5% CO_2 using Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS (Atlanta Biologicals), 4.5 g/mL glucose, 2 mM L-glutamine, 100 U/mL penicillin and 100 U/mL streptomycin (Invitrogen). U937 cells were maintained in complete RPMI medium containing 10% FBS, 2mM L-glutamine, 100 U/mL penicillin and 100 U/mL streptomycin. The R1-null mutant HSV1 ΔICP6 and its parental strain KOS were kindly provided by Dr. Sandra Weller (University of Connecticut Health Center, Farmington, CT). HSV viral stocks were prepared by infecting HT-29 cells stably expressing ICP10 or ICP6 at an MOI of 0.01 and were titered by plaque assay on monolayer cultures of Vero cells. MCMV K181 and MCMV-M45*mut*RHIM viruses were generated as described (Upton et al., 2010). z-VAD-fmk was from Enzo Life Sciences, RIP1 inhibitor GSK'963, RIP3 inhibitor GSK'840 and IAP antagonist SMAC007 (Mandal et al., 2014), as well as the pRIP1 S166-specific antibody, were provided by GlaxoSmithKline (contact Peter Gough, peter.j.gough@gsk.com), IAP antagonist BV6 was provided by DomogojVucic (Genentech), and recombinant human TNF was from R&D.

Plasmids, transfection and transduction

The ICP6 ORF was PCR-amplified from pKHF plasmid (a gift from Dr. Sandra Weller) and then inserted into pCMV10-3XFLAG vector (Sigma) in-frame with an amino-terminal epitope tag. Truncation mutants were generated from pCMV10-3XFLAG-ICP6 as a template using PCR to amplify nucleotides 1-1467, 1-1887, 1-2505, 1-3318, 1-3348, 730-1887 or 730-3414, to produce ICP6(1-489), ICP6(1-629), ICP6(1-835), ICP6(1-1106), ICP6(1-1116), ICP6(244-629) and ICP6(Δ1-243), respectively. ICP6*mut*RHIM was generated by overlap extension PCR to change ICP6 aa73-76 from VQCG to AAAA. ICP6(G865/867/870A) was generated by overlap extension PCR to change ICP6 aa865, aa867 and aa870 from G to A. All plasmids were verified by DNA sequencing. Expression vectors encoding Myc-tagged or FLAG-tagged RIP1, RIP2, RIP3, RIP4 have been previously described (Kaiser and Offermann, 2005; Kaiser et al., 2008; Meylan et al., 2004; Rebsamen et al., 2009). The RIP1 intermediate domain (aa301-558) was PCR amplified and cloned into the expression vectors pcDNA3-6XMyc and pCMV10-3XFLAG. To mutate the RHIM in RIP3 or in RIP1, the amino acids VQVG or IQIG, which correspond to RIP3aa458-461or RIP1aa539–542, were changed to AAAA by overlap extension PCR. ICP10 ORF was PCR-amplified from plasmid AdCMV5-GFP-R1 (a gift from Dr. Yves Langelier) and then inserted into pCMV10-3XFLAG vector (Sigma) resulting in an in-frame amino-terminal 3XFLAG epitope tag. This FLAG-tagged ICP10construct was used as a template using PCR to amplify nucleotides 748-3435 (aa250-1144) and produce ICP10(Δ1-249).Epitope-tagged ICP6 and ICP10 and mutant forms were subcloned into pLV-EF1α-MCS-IRES-Puro (Biosettia) andHT-29 cells stably expressing these proteins were generated by lentiviral transduction (Upton et al., 2010).Transfections were performed with Lipofectamine 2000 (Invitrogen) and DNA at a 2:1 ratio in Opti-MEM (Invitrogen).

Supplemental Figures

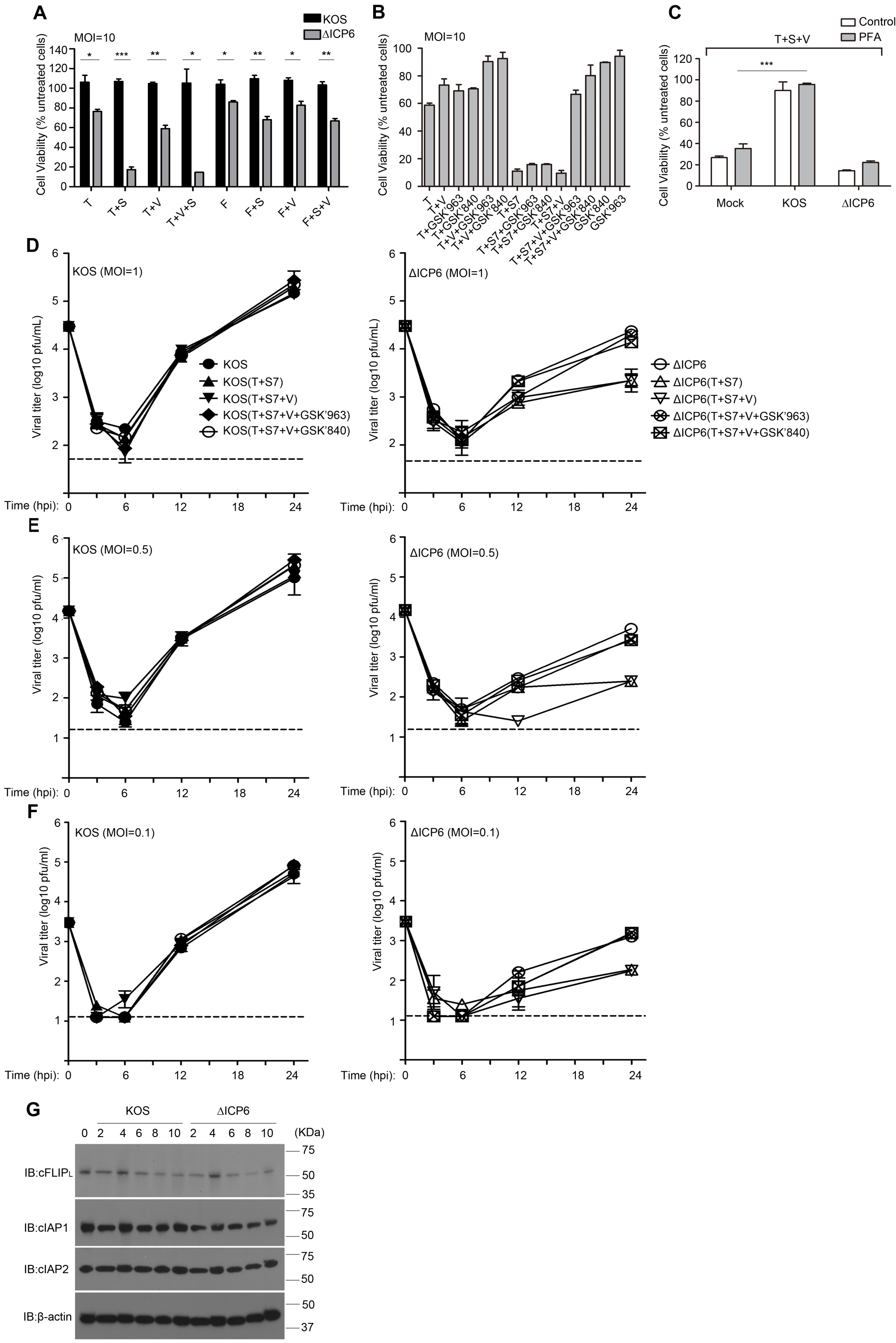
Figure S1, related to Figure 1. (A) Viability of HT-29 cells infected with KOS or ∆ICP6 (MOI=10) and treated from 2 to 14 hpi with T, T+S, T+V, T+S+V or anti-Fas antibody (F; 5 ng/ml), F+S, F+V, F+S+V. (B) Viability of HT-29 cells infected with ∆ICP6 (MOI=10) and treated from 4 to 19 hpi with T, T+V, T+S7, T+S7+V, in the absence or presence GSK'963 (1 μM) or GSK'840 (3 μM). (C) Viability of HT-29 cells infected with mock, KOS, or ∆ICP6 and treated with T+S+V from 2 to 24 hpi in the presence or absence of phosphonoformate (PFA; 300 μg/ml). (D-F) Replication levels of KOS (left) and ∆ICP6 (right) virus in HT-29 cells (D, MOI=1; E, MOI=0.5; F, MOI=0.1) in the absence (DMSO control) or presence of T+S7, T+S7+V or T+S7+V with RIP1 kinase inhibitor GSK'963(1 μM) or RIP3 kinase inhibitor GSK'840 (3 μM), as shown. (G) IB to detect cFLIP, cIAP1, cIAP2 and β-actin in KOS or ∆ICP6-infectedHT-29 cell lysates collected at 2, 4, 6, 8, 10 hpi (MOI=5).

Figure S2, related to Figure 2. (A) Photomicrographs of HT-29-EV, HT-29-ICP6 and HT-29-ICP10 cell cultures at 0, 4, 8, 12 h post-treatment with T+S+V in the presence of Sytox Green (62.5 nM), a nucleic acid detecting fluorescent dye, assessed on an IncuCyte instrument. Original magnification, 200x. (B) Viability of HT-29-EV or HT-29-M45 cells treated with T+S+V for 24h. (C) Viability of HT-29-EV, HT-29-ICP6 or HT-29-ICP10 cells treated with F+S+V for 24 h. (D) Viability of HT-29-EV or HT-29-ICP10 cells treated with T+S+V or F+S+V as indicated for 12, 24 or 48 h. (E) Viability of U937-EV or U937-ICP10 cells treated with T, T+S, T+V or T+S+V as indicated for 18 h. (F) Viability of 3T3-SA-EV or 3T3-SA-ICP10 cell lines treated for 18 h with T (25 ng/mL) and/or V (25 μM). (G) Viability of 3T3-SA-EV or 3T3-SA-ICP6 cells treated for 18 h with T and/or V. (H) Viability of 3T3-SA-EV or 3T3-SA-M45 cells treated for 18 h with T+V (left) or infected with parental K181 and M45mutRHIM virus (MOI=10) (right). Insets depict IB detection of FLAG-ICP10 or FLAG-ICP6 in the transduced whole cell lysates. (I and J) HSV2 ICP10 interaction with RIP1 and RIP3. IB/IP to detectFLAG-ICP10 interaction with Myc-tagged versions of RIP1, RIP1(301-558) intermediate domain, RIP2, RIP3, and RIP4. The top panel shows 293T cell lysates subjected to anti-Myc IP followed by anti-FLAG and anti-Myc IB analysis. (K) Reciprocal IP to detect ICP10 interaction with RIP1 and RIP3. Lysates of cells transfected with Myc-RIP1 or Myc-RIP3, with or without FLAG-ICP10 subjected to IP with anti-FLAG antibody, followed by IB with anti-Myc or anti-FLAG antibody. (L) IP/IB to detect Myc-ICP10 interaction with FLAG-tagged versions of RIP1, RIP1(301-558) and RIP1(301-558, *mut*RHIM). Lysates were subjected to IP with anti-FLAG antibody followed by IB with anti-Myc or anti-FLAG antibody. (M) IP/IB to detect HA-ICP10 with FLAG-tagged RIP3 or RIP3(*mut*RHIM). Lysates were subjected to IP with anti-FLAG antibody followed by IB with anti-HA or anti-FLAG antibodies. The lower panels depict IB of input whole cell lysates.

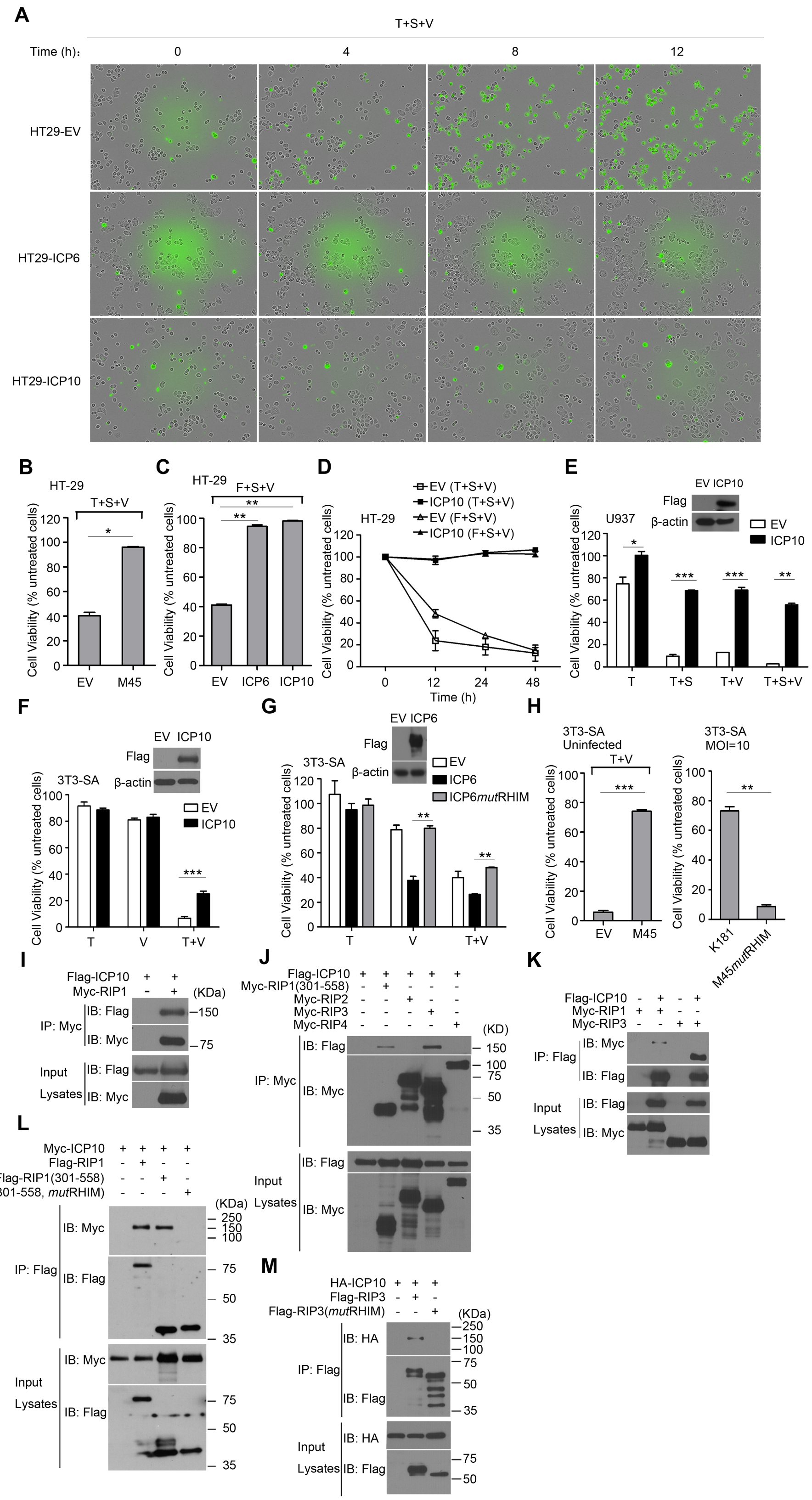
Figure S3, related to Figure 3. (A) IB to detect HT-29 cells stably expressing the indicated FLAG-tagged ICP6 constructs. (B)IP/IB to detect interaction of Myc-tagged ICP6, ICP10, UL45 or M45, or EV, with V5His-Casp8 in transfected 293T cells. Lysates were subjected to IP with anti-Myc antibody followed by IB with anti-His or anti-Myc antibody. The vertical line shows where lanes from the original gel were brought adjacent.

Figure S4, related to Figure 4. (A) Viability of HT-29-EV, HT-29-ICP6, HT-29-ICP6*mut*RHIM and HT-29-ICP6(1-629) cells infected with ∆ICP6 (MOI=5) and treated from 1 to 24hpi with T, T+V, T+S7, in the absence or presence GSK'963 (1 µM) or GSK'840 (3 µM) as shown. (B) Viability of HT-29 cells infected with HSV1 (F strain) or HSV1*mut*RHIM (MOI=10) or mock-infected and then treated from 1 to 24 hpi with T+S7, T+S7+GSK'963, T+S7+GSK'840. (C-E) Viability of HT-29-EV, HT-29-ICP10, HT-29-ICP10(∆1-249) cells, either mock-infected (C) or infected with ∆ICP6 (D) or KOS (E) virus(MOI=5) and treated from 2 to 20 hpi with T, S and/or V. (F) IB to detect HT-29 cells stably expressing FLAG-tagged ICP10, ICP10(∆1-249). (G) IB to detect Casp3 cleavage (Cl-Casp3) and MLKL phosphorylation (p-MLKL) in HT-29 cells stably expressing EV, ICP10(∆1-249), ICP6(∆1-243) and ICP6*mut*RHIM treated with T+S for 8h. (H) Viral plaque numbers of HT-29 cells infected with HSV1 and HSV1*mut*RHIM virus for 72 hs in the absence or presence of T, T+GSK'963 (1 µM), or PFA (150 µg/ml).

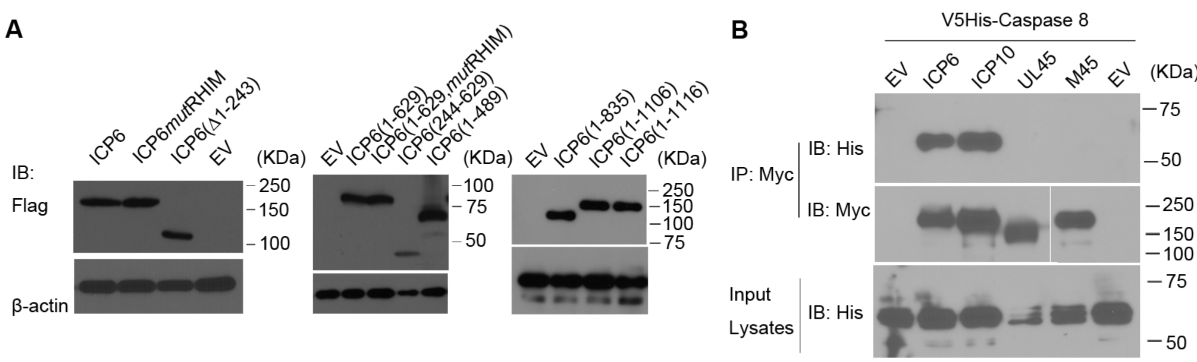
Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3



Supplemental Figure 4